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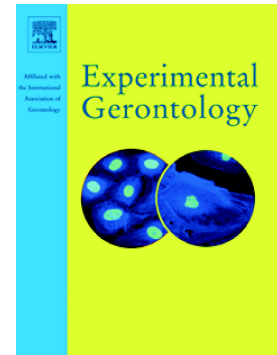
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**Time course of denervation-induced changes in gastrocnemius
muscles of adult and old rats**

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ABSTRACT

Denervation leads to significant muscle atrophy, but it is less clear whether 1) loss of capillaries, fibre size and oxidative capacity decline in parallel and 2) the time course of these changes differs between young and old animals. ~~Little is known, however, about the time course of this atrophy and accompanying changes in the oxidative capacity and capillarisation of the muscle and whether the time course changes with age.~~ To investigate this, we denervated the left gastrocnemius muscle for 1, 2 or 4 weeks, while the right muscle served as an internal control, in rats that were 5- or 25 months old at the end of the experiment. In the fast part of the gastrocnemius muscle, almost all atrophy had occurred after two weeks (42%) of denervation. Even after 4 weeks of denervation, there was no significant reduction in the oxidative capacity of the muscle. Significant capillary loss occurred only after 4 weeks of denervation ($P < 0.001$) that lagged behind and was less than proportional to the decrease in fibre size. Consequently, the capillary density was elevated ($P < 0.001$). The time course of these morphological changes was similar in the 5- and 25-month-old rats. Comparing these data with those previously published in the soleus muscle from the same animals show that the decrease in oxidative capacity and capillary rarefaction were more pronounced and occurred earlier than in the gastrocnemius muscle, respectively. The time course of capillary loss lagged behind the decrease in fibre size, and combined with the absence of denervation-induced changes in oxidative capacity this resulted in a muscle capillary supply in excess of that expected by the metabolism and fibre size at least during the first 4 weeks after denervation.

Keywords: oxidative capacity; capillary density; atrophy; aging; denervation; disuse

INTRODUCTION

Spinal cord injury, peripheral nerve damage and stroke are major causes of permanent or transient muscle denervation. The incidence of spinal cord injury is low (Ferro et al. 2017), but more than 200,000 people a year incur peripheral nerve injury in the United States (Sabatier and English 2015). These injuries have a significant impact on the quality of life of the affected individual that is primarily attributable to muscle paralysis. As a result of denervation the muscle atrophies (Round et al. 1993) and undergoes many other structural and morphological changes, such as changes in myosin expression (Midrio 2006). In addition, long-term denervation may lead to insulin resistance (Hirose et al. 2001).

In cases of peripheral nerve injury (Maas et al. 2007) ~~and stroke (Kwakkel et al. 2008)~~ reinnervation can occur, but before reinnervation is re-established the muscle continues to deteriorate. Even so, in many cases, such as in spinal cord injury, the functional denervation persists. Thus, interventions, such as those that stimulate reinnervation after peripheral nerve injury (Sabatier and English 2015) ~~or stroke (Kwakkel et al. 2008)~~ or that harness muscle plasticity to prevent or reverse these detrimental impacts of denervation on skeletal muscle will have significant benefits for the patient. One such therapy that has proven to be effective in even long-term denervated muscles is functional electrical stimulation in both human (Carraro et al. 2017) and rodent muscle (Nakagawa et al. 2017). Such therapies may require modifications depending on the duration of denervation as before a new steady state is reached the muscle is still adapting to the denervation and may even be more responsive than after long-term denervation. This larger plasticity is a suggestion we derive from the at least transient rise in satellite cells (Viguie et al. 1997; van der Meer et al. 2011) , ribosomes (Ionasescu et al. 1975) and capillary density (Degens

et al. 2008) in denervated muscles. A good understanding of the time course of changes in muscle properties is thus indispensable to design effective interventions.

This initial state of flux is demonstrated in rodents by the ~50% atrophy after just 2 weeks of denervation in the tibialis anterior, extensor digitorum longus (EDL) and gastrocnemius muscles (Adhihetty et al. 2007; van der Meer et al. 2011). The rate and extent of denervation-induced atrophy may, however, be somewhat less in old age (Carlson et al. 2002; Alway et al. 2003). This is not limited to rodents as the decline in muscle volume after 2 weeks of unilateral leg casting was less in old than young men (Suetta et al. 2009). The rate and degree of atrophy may not only be modified by ageing but also be more pronounced in fast than slow muscles (Dedkov et al. 2003). Even within the same muscle it has been reported that though there was significant type II fibre atrophy, the size of type I fibres was preserved after up to 1 year of denervation (Ashley et al. 2007).

Beside muscle atrophy, long-term denervation is accompanied by capillary loss (capillary rarefaction) (Józsa et al. 1980; Tyml et al. 1999; Borisov et al. 2000). This has potentially important implications as capillaries play an important role in muscle oxygenation, regeneration (Omairi et al. 2016) and insulin sensitivity (Wagenmakers et al. 2016). Interestingly, in the fast EDL muscle capillary rarefaction was accompanied by an increase in the proportion of oxidative fibres (Cebasek et al. 2006), which contradicts our previous observation in the slow soleus muscle where both capillary rarefaction and loss of oxidative capacity occurred (Degens et al. 2008). This discrepancy may be related to the denervation-induced reduction in blood flow in slow but not in fast muscles (Turinsky et al. 1998). The above indicates that type and time course of adaptation of the metabolism and capillary bed may

differ between fast and slow muscles, as well as between muscles from young-adult and old animals.

In a previous study, we found that the time course of denervation-induced changes in fibre size, oxidative capacity and capillary rarefaction was similar in the slow soleus muscles of young-adult and old rats Degens et al (2008). The aim of this study is to assess 1) the time course of changes in fibre size, oxidative capacity and capillary rarefaction in a part of the gastrocnemius muscle that exclusively ~~exists of~~ consists of type IIB/X fibres, and 2) whether the time course of denervation-induced changes differ between both young-adult and old rats. We hypothesise that 1) capillary rarefaction lags behind the denervation-induced atrophy and loss of oxidative capacity in the fast region of the gastrocnemius and 2) that all these changes will occur later and less-pronounced in muscles from old than young-adult rats.

MATERIALS AND METHODS

Animals and denervation

Adult (n=15) and old (n=10) male Wistar rats were used for this study. Rats were housed in pairs with *ad libitum* access to food and water. The environment was maintained at 22 °C on a 12 h-12 h light-dark cycle. Rats were randomly assigned to groups in which the left soleus and gastrocnemius muscles were denervated for 1, 2, or 4 weeks and the right hind limb served as an internal control as described previously (van der Meer et al. 2011). In short, under aseptic conditions, rats were anaesthetised with isoflurane and the branches of the *n. Ischiadicus* supplying the gastrocnemius and soleus muscles, but not the branch to the plantaris muscle, were

cut as close to their entry point to the muscle belly as possible and sewn to the biceps femoris muscle to prevent reinnervation. Rats received a subcutaneous injection of Rimadyl ($0.5 \text{ mg}\cdot\text{kg}^{-1}$) after surgery as an analgesic. Irrespective of the duration of denervation, all young-adult and old animals were 5- and 25 months old at the time of tissue collection, respectively. The animals were sacrificed with an intraperitoneal injection of an overdose of pentobarbital. Then the gastrocnemius muscles were quickly dissected from both limbs, blotted dry, weighed and then immediately frozen in liquid nitrogen and stored at -80°C .

Histology

Serial cross sections ($10 \mu\text{m}$) were cut on a cryostat at -20°C , mounted on slides, air-dried and stored at -80°C for further use. The serial sections were stained for dipeptidyl peptidase IV + alkaline phosphatase to illustrate capillaries (Degens et al. 1992). Serial sections were stained for myosin ATPase after pre-incubation at pH 4.6 to distinguish type I and type II fibres, and succinate dehydrogenase (SDH) as an estimate of oxidative capacity, as described previously (Wüst et al. 2009). The myosin ATPase staining was used to select a region of interest that contained solely type IIX/B fibres. In each region of interest at least 50 fibres were analysed. Representative images are shown in figure 1.

Fig. 1 & 2 *Capillarisation*

The method of capillary domains was used to analyse the capillarisation in skeletal muscle (Degens et al. 1992; Wüst et al. 2009). Figure 2 illustrates the analysis. First, capillary coordinates and fibre outlines on the image of stained sections were recorded with BTablet (BaLoH Software, www.baloh.nl). The coordinates of

capillaries and fibre outlines were then imported into AnaTis (BaLoH Software, www.baloh.nl) to calculate capillary domains. A capillary domain is an area surrounding a capillary delineated by equidistant boundaries from surrounding capillaries (Hoofd et al. 1985) and is a good estimate of the capillary oxygen supply area (Al-Shammari et al. 2014). The program also calculates the fibre cross-sectional area (FCSA), fibre perimeter and shape (atrophied fibres may become more angular (Edwards and Jones 1983), capillary density (CD) and capillary to fibre ratio (C:F). The standard deviation of log transformed domain areas (\log_DSD) was used as an index for heterogeneity of capillary spacing, which is a major factor for tissue oxygenation. In addition, the percentage of ~~connective tissue~~ non-contractile material was calculated as the percentage area of the region of interest not covered by fibres. To account for ~~connective tissue~~ non-contractile material, the programme also calculates other indices of capillarisation. The number of domains overlapping a fibre (DAF) is similar to the more commonly used 'capillaries around a fibre'. The local capillary to fibre ratio (LCFR) of a given fibre is given by the sum of domain fractions overlapping that fibre, and takes into account that a capillary supplies more than one fibre. The capillary fibre density (CFD) of a given fibre) was calculated as the LCFR divided by the FCSA.

Oxidative capacity

The maximal oxygen consumption of muscle fibres was determined as described previously (van der Laarse et al. 1989; Des Tombe et al. 2002). The optical density of SDH-stained fibres at 660 nm (OD_{660}) was determined with ImageJ (ImageJ; NIH, USA). For each section, a separate calibration curve was constructed with a series of filters with a known optical density to prevent bias related to differences in

background staining intensity and lighting between sections and over time, respectively. The mass-specific fibre maximal oxygen consumption ($VO_{2\max_{\text{mass-specific}}}$ in $L \cdot kg^{-1} \cdot min^{-1}$) was calculated (assumption that one mole of oxygen is 22.4 litres and the density of muscle is $1 \text{ kg} \cdot L^{-1}$) as:

$$VO_{2\max} = 0.672 \times SDH_OD$$

Where SDH_OD is the optical density of the SDH stain. The $VO_{2\max_{\text{fibre}}}$ was calculated as $VO_{2\max_{\text{mass-specific}}} \times FCSA$ and gives the maximal oxygen consumption of a fibre in $pL \cdot mm^{-1} \cdot min^{-1}$ (Bosutti et al. 2015).

Statistics

Two-way analysis of variance (ANOVA) was used to determine the effects of age and duration of denervation. The factor age had 2 levels (adult or old), and the factor duration of denervation had 4 levels (0, 1, 2, and 4 weeks denervation). An age * duration interaction indicates that the effect of denervation differed between age groups. Only if such an interaction was found, an ANOVA was applied to each age group separately with a Bonferroni-corrected post-hoc test, while differences between the young and old control groups were tested with a t-test. If a main effect of duration of denervation was found and no interaction, Bonferroni-corrected post-hoc tests were performed on the pooled young-adult and old data to locate the differences between time-points. Effects were considered significant at $P < 0.05$. All data are presented as mean \pm SD.

Table 1

RESULTS

The data on body mass and gastrocnemius muscle mass have been reported before (van der Meer et al. 2011), but are given here for completeness (Table 1). In short,

the gastrocnemius muscle mass decreased during the first 2 weeks of denervation and then reached a stable level. The ratio of muscle mass to body mass was lower in old compared to adult rats at all time points because of the higher body mass of old rats, except at 4 weeks of denervation.

The ATPase staining confirmed that all fibres in the parts of the muscle we studied were indeed type IIB/X fibres (Fig. 1). For none of the morphological parameters, with the exception of C:F/(FCSA \times SDH), significant age \times denervation interactions were found. This indicates that the time course denervation was similar in young-adult and old rats.

Table 2 The percentage of non-contractile material/tissue was not significantly affected by age ($P=0.868$). The percentage of non-contractile material/tissue was higher than control after one and four weeks of denervation ($P<0.05$; Table 2).

Fig. 3 Figure 3a shows the changes in FCSA during denervation. The FCSA was larger in old than young-adult gastrocnemius muscles ($P=0.028$; Fig. 3a) and was reduced after 2 weeks of denervation ($P<0.05$) with no significant further decline between 2 and 4 weeks of denervation (Fig. 3a). The shape of the fibres, as indicated by the roundness index, was neither significantly affected by age ($P=0.088$), nor by the duration of denervation ($P=0.053$) (Table2).

The C:F was higher in muscles from old than those from young-adult rats ($P<0.001$; Fig. 3b). The main-effect of denervation ($P=0.017$) was evident as a reduced C:F ratio after 4 weeks of denervation in both young-adult and old animals (Fig. 3b).

The CD in young-adult and old rats did not differ significantly ($P=0.526$; Fig. 3c). The effect of the duration of denervation ($P<0.001$) was apparent as a higher CD after 2 and 4 weeks of denervation than in the contra-lateral control muscles (Fig. 3c).

The variation in the size of the fibres, expressed as $FCSA_{SD}$, was larger in old than young-adult animals ($P=0.003$) and was less after 2 and 4 weeks of denervation ($P<0.05$) than in the contra-lateral control leg (Table 2).

The heterogeneity of capillary spacing, indicated as Log_DSD , was not significantly affected by age ($P=0.221$), nor duration of denervation ($P=0.089$).

Fig. 4 The DAF (Fig. 4a) and LCFR (Fig. 4b) followed the same pattern as the C:F ratio and were both higher in muscles from old than young-adult rats ($P<0.001$). Both DAF and LCFR were, like C:F, reduced after 4 weeks of denervation ($P<0.01$). The CFD followed the same pattern as CD and was elevated after 4 weeks of denervation ($P=0.001$), and did not differ significantly between young-adult and old rat gastrocnemius muscles.

Fig. 5 The $VO_{2max_{mass\ specific}}$ of the fibres was not significantly affected by age ($P=0.223$) or duration of denervation ($P=0.318$) (Fig. 5A). For $VO_{2max_{fibre}}$ there was no significant effect of age ($P=0.069$), but the $VO_{2max_{per\ fibre}}$ was less than control after 4 weeks of denervation ($P=0.004$; Fig. 5B). Since the capillary supply to a fibre is determined by both oxidative capacity and fibre size, we calculated the $C:F/(FCSA \times SDH)$, taking both the size and the oxidative capacity of the fibres into account. For the $C:F/(FCSA \times SDH)$ we found not only significant age ($P=0.004$) and duration effects ($P<0.001$), but also an age * duration interaction ($P=0.014$), indicating that the effect of denervation on this parameter differs between young-adult and old rats. The $C:F/(FCSA \times SDH)$ was elevated at 1, 2 and 4 weeks of denervation in young-adult

($P < 0.05$), but not in old rats (Fig. 5c). There was no significant difference in the C:F/(FCSAxSDH) between young and old control muscles (Fig. 5C).

DISCUSSION

The main observation of this study was that denervation-induced loss of capillaries also known as capillary rarefaction, ~~capillary rarefaction~~ was only significant after 4 weeks of denervation in the fast region of the rat gastrocnemius muscle (further referred to for convenience as gastrocnemius muscle), while the bulk of muscle fibre atrophy had already occurred after 2 weeks. Capillary rarefaction thus lagged behind fibre atrophy, and hence denervated rat gastrocnemius muscle had a denser capillary network. The denser capillary network and unchanged oxidative capacity resulted in a capillarisation in excess of oxidative capacity, at least during the first 4 weeks after denervation. The time course of these denervation-induced changes was similar in young-adult (5 months old) and old (25 months old) rats, and similar to that observed in the soleus muscle of the same animals (Degens et al. 2008).

Ageing

In the present study, the gastrocnemius muscle of 25-month-old rats had larger fibres than those of 5-month-old rats. At first glance this seems at odds with other studies that showed an age-related atrophy in rat muscles and particularly so in the glycolytic - that we studied here – and not in the oxidative region of the gastrocnemius muscle (Hepple et al. 2004). However, rats continue to grow during the first year of life, and muscle fibres are indeed larger in 1-year-old than 5-month-old rats (Degens et al. 1993; Lushaj et al. 2008). After reaching a plateau, it is only

after the age of 22 months that fibres (Lushaj et al. 2008), especially type IIB/X fibres (Degens et al. 1993) that we studied here, decrease in size.

The larger fibre size in gastrocnemius muscles from the 25-month-old than 5-month-old rats was associated with a higher capillary to fibre ratio (C:F), as reflected by a similar capillary density. As there was no difference in the oxidative capacity of the muscle, this indicates that the coupling between fibre size and capillary supply to a fibre (Wüst et al. 2009) was similar also in young-adult and old muscle regions consisting solely of type IIB fibres.

The explanation for the absence of differences in muscle morphology in gastrocnemius muscles from 5- and 25-month-old rats may be related to the fact that we compared still growing young-adult rats with animals that show the first signs of sarcopenia, similar to comparing the muscles of a 17-year-old with a 65-year-old man (Ballak et al. 2014). In line with our previous observation in the plantaris muscle of the same animals, we found that also in the region of the gastrocnemius muscle that contains only type IIB/X fibres the variation in fibre size was larger in 25- than 5-month-old rats (Degens et al. 2009). The 25-month-old animals are thus showing early signs of ageing, before the development of sarcopenia and provide thus a model to assess whether the response to denervation was already changed from that observed in young-adult rats during early stages of muscle ageing. This is important as a significant part of the age-related muscle wasting is thought to be due to a slow but progressive denervation-reinnervation process (Larsson and Ansved 1995; Degens and McPhee 2014) and allows us to compare the response to denervation in muscles that are still growing and those that already suffer from early signs of sarcopenia.

Denervation

Similar to previous studies on soleus muscles (Ansved and Larsson 1990; Degens et al. 2008), we observed that the extent of denervation-induced atrophy of gastrocnemius muscle was similar in adult and old rats and occurred almost entirely during first 2 weeks of denervation. Here we did not observe any significant alteration to the shape of the fibres, something seen in muscle pathologies (Edwards and Jones 1983) suggesting that only after more than 4 weeks of denervation such changes may become apparent.

Although changes in muscle mitochondrial density and SDH activity are not always proportional (Mayne et al. 1991) it has been reported to be a great marker of mitochondrial volume density, even in chronic electrically-stimulated muscle (Reichmann et al. 1985). Therefore, the maintained SDH activity indicates that the denervation-induced loss of mitochondria in the gastrocnemius muscle was proportional to the atrophy. In the soleus muscle of the same animals, however, the denervation-induced loss of mitochondria exceeded the atrophy, as reflected by a decreased oxidative capacity of the fibres (Degens et al. 2008). Although a reduction in oxidative capacity has often been reported after denervation (Adihetty et al., 2007; Eisenberg and Hood, 1994), others have even seen an increased, rather than a decreased proportion of oxidative fibres after two weeks of denervation in the fast and highly glycolytic extensor digitorum longus muscle (Cebasek et al. 2007). Also in the vastus lateralis muscle of spinal cord injury patients an elevated oxidative capacity has been reported (Castro et al. 1999). Overall, this suggests that the effects of denervation are muscle dependent, where the reduction in oxidative

capacity is most pronounced in highly oxidative muscles and may even be elevated in fast muscles.

The cause of this different response is not known, but may be related to the role of mitochondria in denervation-induced muscle wasting (Siu and Alway 2005; Adhihetty et al. 2007). It is possible that the production of reactive oxygen species by leaking mitochondria will cause a chain reaction of damage in neighbouring mitochondria, which are in closer vicinity in highly oxidative than glycolytic muscle fibres. There is some evidence that this may be the case as we have found previously that the redox sensitive inhibitor of differentiation 3 (Id-3) protein (Mueller et al. 2002) was elevated more in 2-weeks denervated soleus than gastrocnemius muscles (Alway et al. 2003).

Here we found that denervation-induced capillary rarefaction occurred only after almost all atrophy had already taken place. Consequently, the capillary density in the muscle was elevated, and given that the oxidative capacity was not changed, the denervated muscle had an excess capillary supply. These results are in line with the loss of capillaries and increased capillary density in the extensor digitorum longus muscle that had been denervated for 4 weeks (Čebašek and Ribarič 2016). Previously we found that the C:F in the soleus muscle of the same animals was already reduced after 2 weeks of denervation (Degens et al. 2008). Others have also found that after 2 weeks of denervation capillary rarefaction had occurred in the soleus, but not yet in the extensor digitorum muscles (Cebasek et al. 2006). These observations thus suggest that slow muscles show a faster denervation-induced loss of capillaries than fast muscles.

Such a different time course in capillary rarefaction between slow and fast muscles may be related to differences in the denervation-induced changes in blood flow.

Endothelial shear stress, caused by flow of blood, is an important factor for the maintenance of the capillary bed (Hudlicka et al. 1992). The denervation-induced decrease in resting blood flow in soleus and increase in gastrocnemius (Turinsky et al. 1998) and fast tibialis anterior muscle up to 21 days post denervation (Eisenberg and Hood 1994) may thus underlie the delayed capillary rarefaction in the fast muscles. Whatever the cause, both the denervated soleus (Degens et al. 2008) and gastrocnemius muscle have a capillary supply in excess of that expected from the size and oxidative capacity of the muscle fibres, particularly in young-adult, but less so in old denervated muscles. Something similar has also been seen in muscles from rats older (28-30 months old) than those used in the present study (Hepple et al. 2004), in human muscle after bed rest (Bosutti et al. 2016) and in gastrocnemius muscle of moderate to severe denervation in human subjects (Carpenter and Karpati 1982). If the duration of denervation extends beyond 4 weeks further capillary loss may occur, resulting also in a decreased capillary density (Borisov et al. 2000; Dedkov et al. 2003).

The distribution of capillaries in the muscle is rarely considered, but an increased heterogeneity of capillary spacing can have a detrimental impact on muscle oxygenation (Piiper and Scheid 1991; Degens et al. 2006). Here we found that the capillary rarefaction in the denervated muscles did not alter the heterogeneity of capillary spacing. This suggests that the capillary loss, like the angiogenesis during hypertrophy where the heterogeneity of capillary spacing was also unaltered (Degens et al. 1992), is not random, but somehow controlled for adequate oxygen supply and/or other functions of the microcirculation such as removal of heat and metabolic waste, and delivery of energy substrates, such as glucose. After long-term denervation, however, the heterogeneity of capillary spacing was found to increase

(Borisov et al. 2000). The authors suggested that this may be due to spatial separation of capillaries from fibres by deposition of collagen, something that might have been in the initial stages in our study where we did observe an increased proportion of ~~connective tissue~~ non-contractile material in the denervated muscles.

Interestingly, the time course and magnitude of the denervation-induced changes in the gastrocnemius muscle were, like those in the soleus (Degens et al. 2008), largely similar in young-adult and old rats. This is somewhat unexpected, as it has been shown that the response to chronic electrical stimulation is delayed (Walters et al. 1991) and the hypertrophic response is attenuated (Degens and Alway 2003) in old rats. The animals in these latter studies were older (≥ 26 months old) than the old animals in our study, and given that the rate of muscle ageing increases with increasing age (Lushaj et al. 2008), we might have had animals that were just at a very early stage of sarcopenia. ~~In support of having used animals in an early stage of age-related muscle remodelling is the slower loss of capillaries in the denervated soleus muscles from old than young-adult rats (Degens et al. 2008) and the larger variation in fibre sizes in their plantaris muscles (Degens et al. 2009).~~

Previously, we observed that the number of satellite cells was transiently elevated during the first week of denervation (van der Meer et al. 2011). Also the ribosomal content, indicative for an enhanced capacity for protein synthesis, is transiently elevated (Ionasescu et al. 1975). All these observations suggest that there is a window of opportunity where the denervation-induced adaptations are conducive to respond to interventions designed to recover from denervation-induced atrophy, and starting an intervention early may thus have a much larger benefit than starting a similar intervention much later. Indeed, the restorative capacity of the muscle has

been reported to be decreased after long-term denervation (Viguie et al. 1997). The muscle wasting during many systemic diseases shows similar transcriptional changes to that seen during denervation-induced atrophy and our observations may thus have wider implications than just denervation-induced muscle wasting caused by stroke, spinal cord injury and peripheral nerve injury alone.

In conclusion, our study shows that in the fast compartment of the gastrocnemius muscle the time course of denervation-induced changes is similar in young-adult and old rats. Despite capillary rarefaction, the denervated muscles are characterised by a capillary supply in excess of that expected from fibre size and oxidative capacity, at least up to 4 weeks after denervation. Comparing the present data in the gastrocnemius muscle with previous data for the soleus muscle from the same animals (Degens et al. 2008) indicates that the loss of capillaries and reduction in oxidative capacity occur earlier in slow than fast muscles or fast muscle compartments.

Conflict of interest

None declared.

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Figure legends:

Fig. 1 Serial sections from young-adult (5 months old) (**a-c**) and old (25 months old) (**d-f**) control and 4-week denervated (**g-i**) old (25 months old) rat gastrocnemius muscles stained for capillaries (**a,d,g**), myosin ATPase (**b,e,h**) and succinate dehydrogenase (**c,f,i**). Scale bar denotes 100 μ m.

Fig. 2 Illustration of the analysis of capillarisation by the method of capillary domains (**a**) typical example of a section stained to identify capillaries (**b**) fibre outlines in green and capillaries in red (**c**) the calculated capillary domains in blue (**d**) overlaps of domains and fibres; highlighted in red is a fibre and in blue domains supplying that individual fibre; arrows indicate corresponding capillaries and + denotes the same fibre on each panel.

Fig. 3 Changes in the (**a**) fibre cross-sectional area (FCSA) (**b**) capillary-to-fibre ratio (C:F) (**c**) anatomical capillary density (CD) in 5- and 25-month-old rats after 1, 2 and 4 weeks of denervation. There were no significant age * denervation interactions for FCSA ($P=0.711$), C:F ($P = 0.567$) and CD ($P=0.642$) indicating that the effects of denervation were similar in young-adult and old rats. For FCSA main effects of age ($P=0.028$) and duration of denervation ($P<0.001$) were found. There were effects of age ($P<0.001$) and duration of denervation ($P=0.017$) on C:F. There was no significant effect of age on CD ($P=0.526$), but there was an effect of the duration of denervation ($P<0.001$). *: difference between young-adult and old rats; ^a: different from control; ^b: different from 1 week denervation, all at $P<0.05$

Fig. 4 (a) Domains around a fibre (DAF) (b) local capillary to fibre ratio (LCFR) and (c) capillary fibre density (CFD) in gastrocnemius muscles from 5- and 25-month-old rats after 1, 2 and 4 weeks of denervation. There were main effects of age ($P<0.001$) and denervation ($P<0.05$) for DAF, LCFR. For CFD no significant age effect was found, but there was a main effect of denervation ($P=0.001$). There were no significant age * denervation interactions for DAF, LCFR and CFD. *: difference between adult and old rats; ^a: different from control; ^b: different from 1 week denervation, all at $P<0.05$

Fig. 5 (a) Mass specific maximal oxygen consumption ($VO_{2max_{mass\ specific}}$) (b) maximal oxygen consumption of a fibre ($VO_{2max_{fibre}}$) and (c) capillary to fibre ratio per optical density of succinate dehydrogenase stain and cross-sectional area of the fibres ($C:F/(SDH \times FCSA)$) in the gastrocnemius muscle from 5- and 25-month-old rats after 1, 2 and 4 weeks of denervation. The $VO_{2max_{mass\ specific}}$ was not significantly affected by age ($P=0.223$) or duration of denervation ($P=0.318$) and neither was there a significant age * denervation interaction ($P=0.282$). For $VO_{2max_{fibre}}$ there was a main effect of duration of denervation ($P=0.004$), but no age effect ($P=0.069$) or age * duration interaction ($P=0.316$). For the $C:F/(SDH \times FCSA)$ there was an ~~age~~ significant age ($P=0.004$) and denervation effects ($P<0.001$) as well as an age * duration interaction ($P=0.014$). ^a: different from control at $P<0.05$

Table 1 Effect of denervation on gastrocnemius muscle mass and gastrocnemius mass normalised to body mass (GM:BM) in young-adult (A) and old (O) rats, as published previously (van der Meer et al. 2011) but given here for completeness.

	Body mass (g)		Muscle mass (mg)		GM:BM (mg·g ⁻¹)	
Duration of Denervation (n, A /n, O)	A	O	A	O	A	O
0 WEEK(15/10)	442±20	607±57	2231±135	2218±225	5.1±0.3	3.7±0.2 [*]
1 WEEK (5/3)	444±13	584±18	1724±63 ^a	1624±173 ^a	3.9±0.2 ^a	2.8±0.3 ^{a,*}
2 WEEKS (5/3)	443±14	610±64	1269±44 ^{ab}	1313±117 ^a	2.9±0.1 ^{ab}	2.2±0.1 ^{a,*}
4 WEEKS (5/4)	439±31	621±77	732±119 ^{ab}	1174±363 ^a	1.7±0.3 ^{ab}	1.9±0.4 ^{ab}
ANOVA EFFECTS						
AGE	P<0.001		NS		P<0.05	
DURATION	NS		P<0.05 ^a and P<0.05 ^b		P<0.05 ^a and P<0.05 ^b	
INTERACTION	NS		P=0.01		P<0.001	

n: number of animals; body mass values at 0 week are the average from all adult and old animals at the end of the experiment; NS: not significant; P values indicate main effects; unless an interaction effect was found, in which case the P values indicate the results of post hoc tests; ^a: significantly different from control; ^b: significantly different from 1 week of denervation; ^{*}: different from young-adult (P<0.05).

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Table 2 The percentage of non-contractile material, roundness index of the muscle fibres and heterogeneity of capillary spacing (Log_{10}SD) in gastrocnemius muscles from 5- (young-adult) and 25-month-old (old) rats denervated for 1, 2, or 4 weeks.

Duration of Denervation (n, A /n, O)	% Non-Contractile Tissue		Roundness index		FCSA_{SD}		Log_{10}SD	
	Adult	Old	Adult	Old	Adult	Old	Adult	Old
0 WEEK (15/10)	8.7±1.8	7.8±1.2	1.30±0.10	1.37±0.10	691±219	966±397 *	0.164±0.017	0.168±0.016
1 WEEK (5/3)	10.2±1.9 ^a	12.4±5.1 ^a	1.37±0.06	1.44±0.06	550±49	772±231 *	0.154±0.014	0.161±0.018
2 WEEKS (5/3)	10.4±2.0	10.4±1.1	1.42±0.06	1.45±0.12	303±127 ^a	709±183 ^a *	0.157±0.015	0.155±0.005
4 WEEKS (5/4)	14.1±1.3 ^a	12.2±4.4 ^a	1.38±0.06	1.43±0.11	255±37 ^{ab}	437±244 ^{ab} *	0.142±0.011	0.159±0.017

A: young-adult; O: old; % non-contractile tissue was not significantly affected by age ($P=0.868$), but was affected by the duration of denervation ($P<0.001$). There was no significant age * duration interaction. The roundness index was not affected by age ($P=0.088$) nor by the duration of denervation ($P=0.053$). The FCSA_{SD} of fibres was significantly affected by both age ($P=0.003$) and

duration of denervation ($P < 0.001$). No significant age * duration interaction was found. The Log_{10}SD was neither affected by age ($P = 0.221$) nor duration of denervation ($P = 0.089$); ^a: different from control; ^b: different from 1 week denervation; *: difference between adult and old rats, all at $P < 0.05$

Highlights:

- Time course of denervation-induced changes in the fast part of the gastrocnemius muscles of 5- and 25-month-old rats was assessed during the first 4 weeks after denervation.
- The time course of denervation-induced morphological changes was similar in the adult and old rats.
- Despite capillary loss, the denervated muscles are characterized by a capillary supply in excess of that expected from fiber size and oxidative capacity, at least up to 4 weeks after denervation.
- Capillary loss and reduction in oxidative capacity occur earlier in slow than fast muscles.

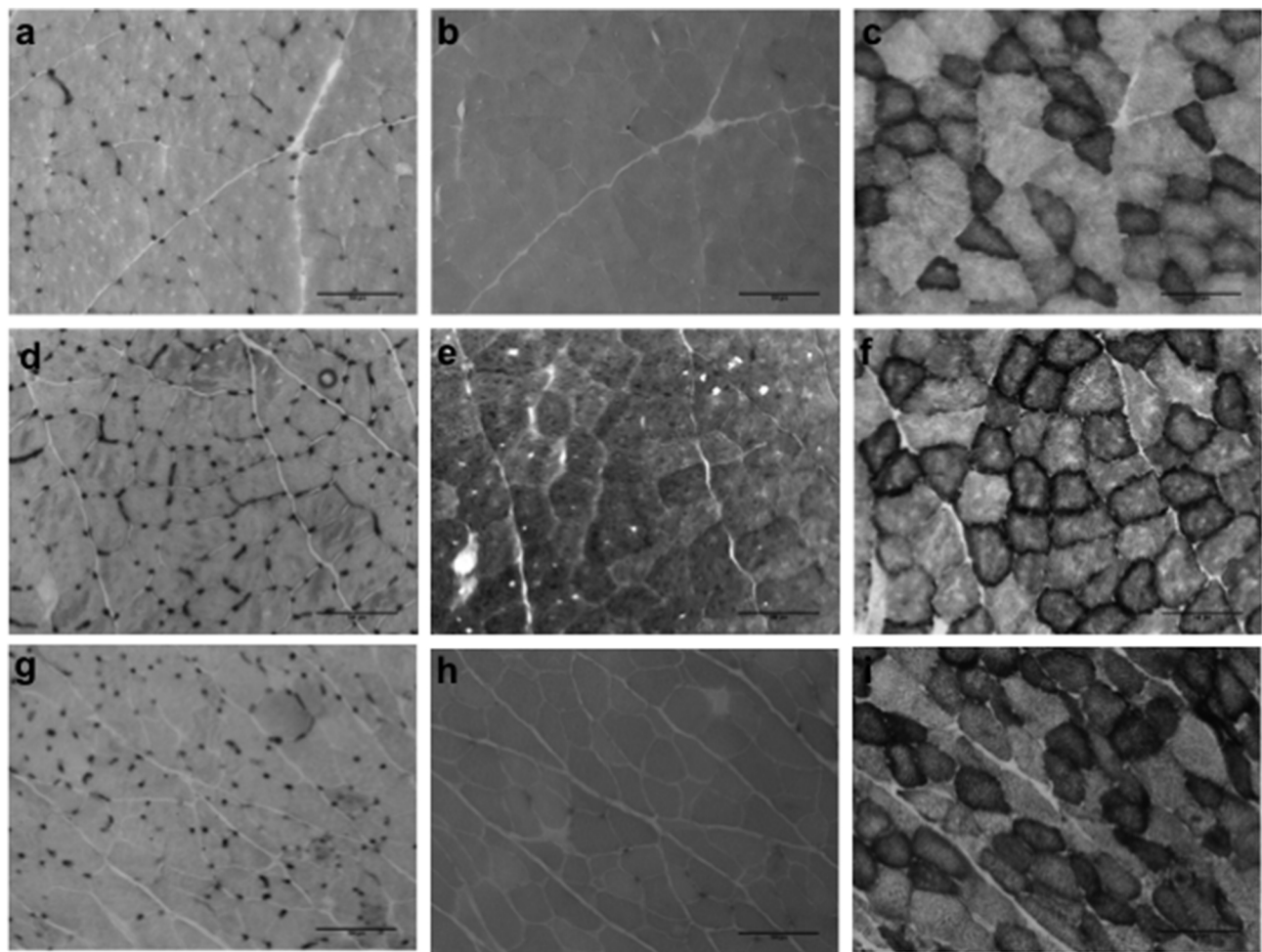


Figure 1

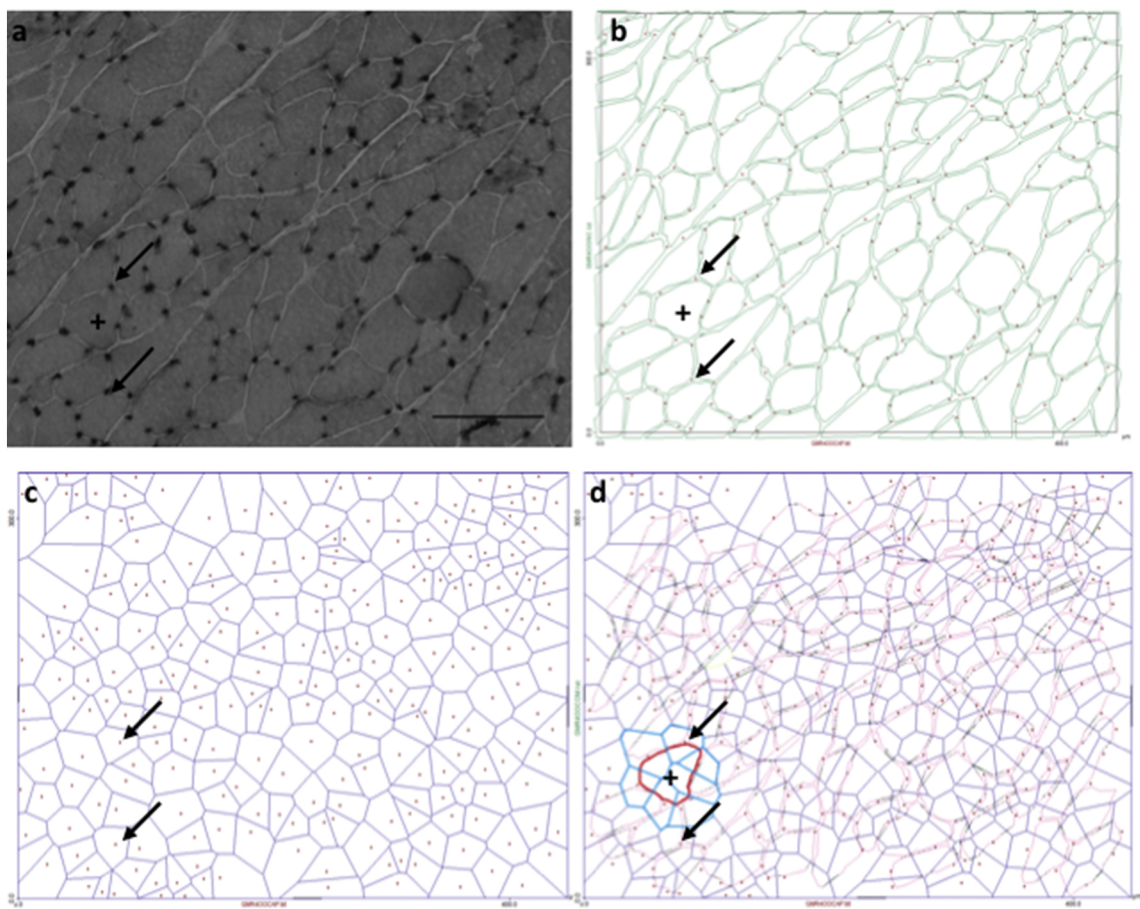


Figure 2

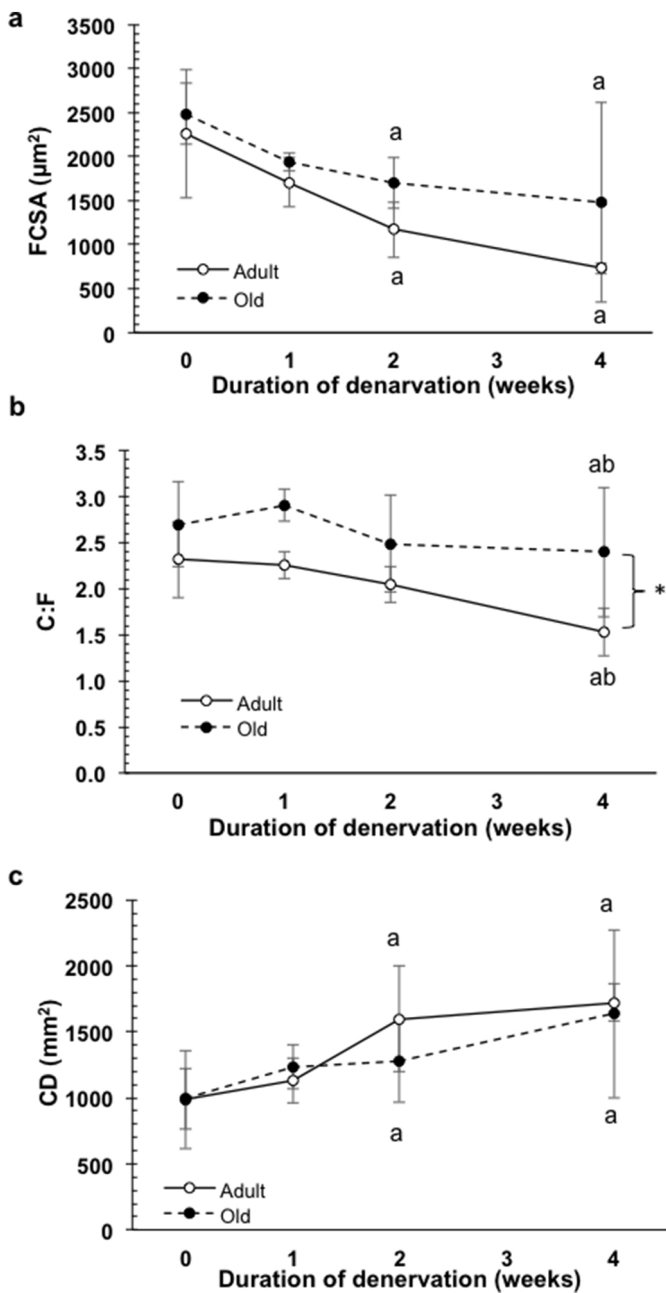
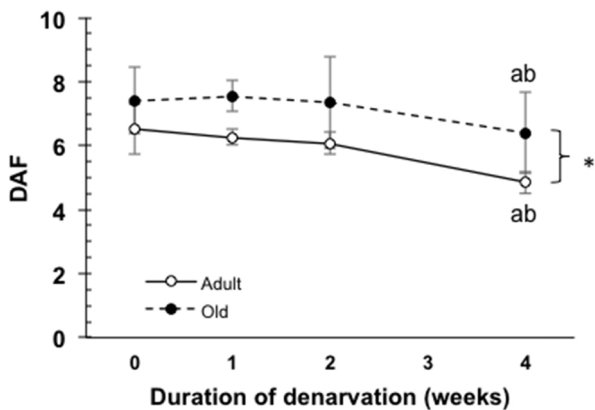
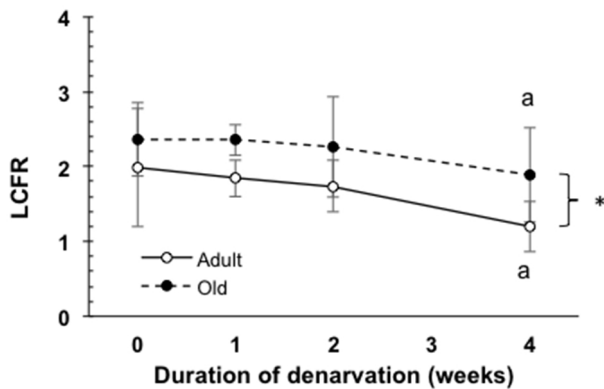


Figure 3

a



b



c

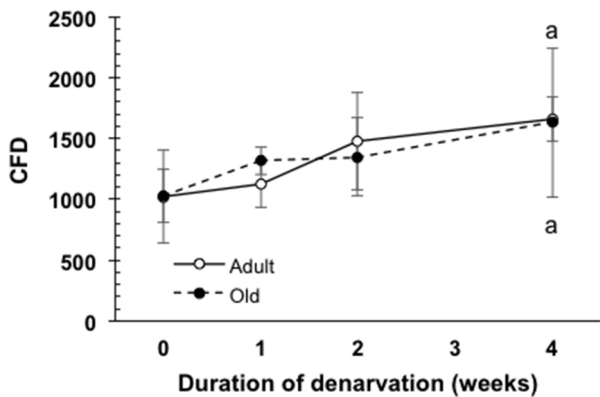


Figure 4

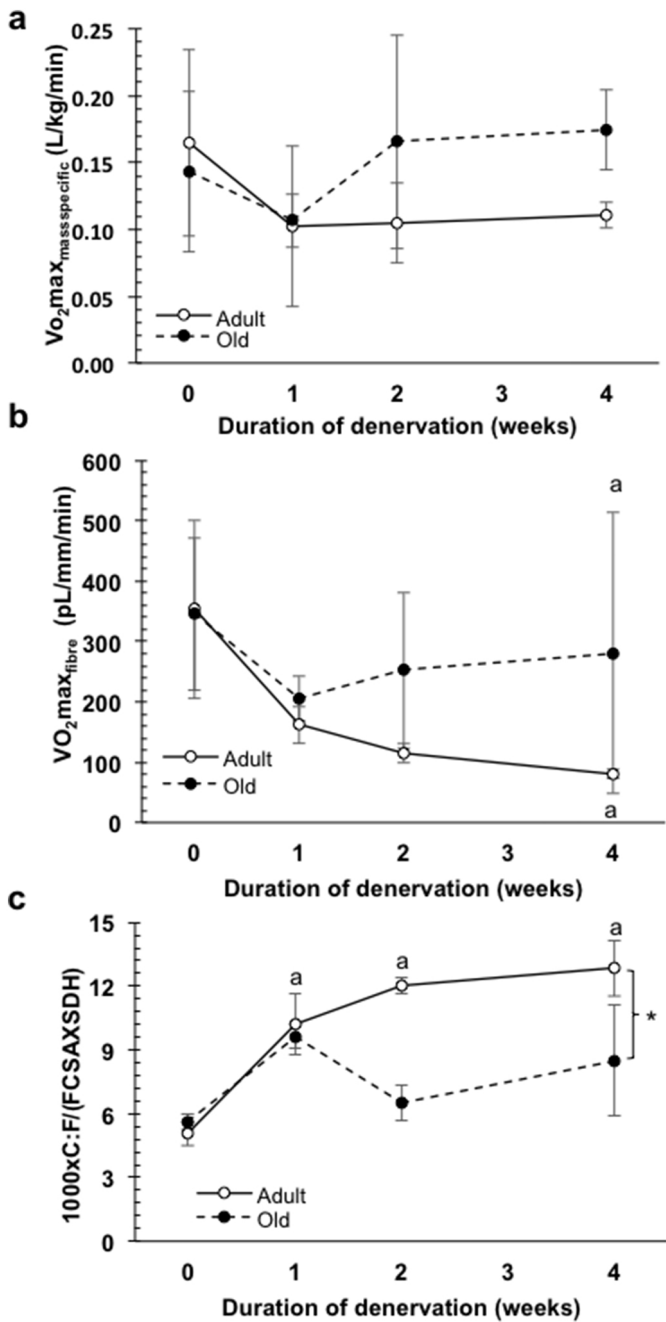


Figure 5